β_2 MICROGLOBULIN FUSION PROTEINS AND HIGH AFFINITY VARIANTS

Field of the Invention

This invention relates to compositions based on β_2 microglobulin, and the use of such compositions in immunological methods pertaining to the targeting of proteins to cell surfaces. The disclosed compositions and methods have particular application to vaccination and tumor therapy.

Background of the Invention

MHC I and activation of cytotoxic T-cells

The beta-2 microglobulin (β_2m) protein is a component of the class I major histocompatibility complex (MHC I). MHC I is formed by the association of β_2m and an alpha protein (also known as the "heavy" chain), which comprises three domains, a1, a2 and a3. MHC I is found on the surface of most types of nucleated cells, where it presents antigens derived from proteins synthesized in the cytosol to $CD8^+$ T-cells. Two signals are required for activation of naive $CD8^+$ T-cells. The first signal is provided by the interaction of the T-cell receptor (TCR) with the MHC I-antigen complex on the antigen-presenting cell surface. The second signal is generated by the interaction of a ligand on the antigen-presenting cell (APC) with a second receptor present on the T-cell surface. This second signal is termed co-stimulation, and the APC ligand is often referred to as a co-stimulatory molecule. The best characterized co-stimulatory molecules on APCs are the structurally related glycoproteins B7.1 (CD80) and B7.2 (CD86) which interact with the CD28 receptor on the T-cell surface. Activation of $CD8^+$ T-cells by these two signals leads to the proliferation of antigen-specific cytotoxic T-cells, which recognize and destroy cells presenting the signaling antigen. These cytotoxic T-cells play an important role in the immunological defense against intracellular pathogens such as viruses, as well as tumors. A detailed presentation of the immunological basis of the cytotoxic T-cell response can be found in Janeway and Travers (*Immunobiology: the immune system in health and disease*, Current Biology Ltd./Garland Publishing, Inc. New York, 1997).

The failure of an exogenous (non-self) antigen to stimulate a cytotoxic T-cell response can result from a block in the above-described cytotoxic T-cell activation pathway at one of many points (see Ploegh, 1998, *Science* 280:248-53). Failure of the cytotoxic T-cell activation pathway is of great significance in two particular areas of medicine: vaccination and tumor immunology.

Cytotoxic T-cells and vaccination

Vaccine technology has focused in recent years on sub-unit vaccines. Sub-unit vaccines comprise isolated pathogen components, such as viral capsid or envelopes, or synthetic peptides that mimic an antigenic determinant of a pathogen-related protein. For example, U.S. Patent No. 4,974,168 describes leukemia associated immunogens that are peptides based on envelope proteins of

a leukemia-associated virus. However, while sub-unit vaccines can stimulate $CD4^+$ helper T-cells (which play a key role in humoral immunity), attempts to stimulate $CD8^+$ cytotoxic T-cells *in vivo* with such vaccines have been largely unsuccessful. It has been postulated that the reason for this is the inability of the exogenously administered vaccine peptide to associate with the MHC I molecules on the cell surface (Liu, 1997, *Proc. Natl. Acad. Sci. USA* 94:10496-8). In other words, the block in the cytotoxic T-cell activation pathway occurs at the stage where the antigen is loaded into the MHC I molecule.

One proposed solution to this problem is to combine the antigenic peptide with a molecule that is readily taken up into cells (reviewed by Liu, 1997, *Proc. Natl. Acad. Sci. USA* 94:10496-8). Thus, this strategy is based on getting the antigen into the cytosol so that it can join the normal pathway by which antigens are processed for presentation by MHC I. In contrast, Rock et al. (*J. Immunol.* 150:1244-52, 1993) adopted a strategy of enhancing the binding of the vaccine peptide to MHC I already present on the cell surface. Rock et al. (*J. Immunol.* 150:1244-52, 1993) report that the administration of exogenous purified β_2m along with the vaccine peptide produces enhanced loading of the peptide onto MHC I *in vivo* and thereby stimulates a cytotoxic T-cell response against the peptide. The use of exogenous β_2m as a vaccine adjuvant is also described in U.S. Patent No. 5,733,550 (to Rock et al.), which is incorporated herein by reference.

Tumor cells and immune system evasion

Tumor cell immunity is primarily cell-mediated, involving both $CD8^+$ cytotoxic T-cells and $CD4^+$ helper T-cells. However, despite the fact that tumor cells express tumor-specific proteins that are not recognized as self-antigens by the immune system, they often escape recognition by the immune system. A number of factors may contribute to the ability of tumor cells to evade immune recognition, including the down-regulation of expression of co-stimulatory proteins. TCR stimulation in the absence of co-stimulatory molecules can result in failure to activate the T-cell and the induction of clonal anergy. Thus, down-regulation of co-stimulatory proteins in tumor cells prevents normal activation of T-cells that do bind to tumor antigens on the cell surface, permitting the tumor cell to escape recognition.

Several research groups have attempted to address this issue by removing tumor cells from a patient, providing exogenous co-stimulatory molecules on the surface of the removed tumor cells and then reintroducing the tumor cells to the patient so that immune recognition can occur. For example, European patent application number 96302009.4 describes a method by which tumor cells are removed from a patient, transfected to express both B7 and CD2 (a co-receptor involved in T-cell adhesion and activation) on the tumor cell surface, and then reintroduced to the patient. The reintroduced cells are reported to stimulate a broad immunological response against both the reintroduced transfected tumor cells and the non-transfected tumor cells within the patient's body, resulting in tumor regression.

Adopting an alternative approach to this problem, Gerstmayer et al. (*J. Immunol.* 158:4584-90, 1997) describe a chimeric B7-antibody protein, in which the antibody is specific for the *erb*B2 proto-oncogene product. This chimeric molecule localizes specifically on the surface of *erb*B2 expressing tumor cells, and presents the B7 co-stimulatory molecule to cytotoxic T-cells, resulting in enhanced proliferation of cytotoxic T-cells. Gerstmayer et al. (*J. Immunol.* 158:4584-90, 1997) thus propose that fusion proteins comprising an anti-tumor antibody and a co-stimulatory molecule could be useful as tumor immunotherapeutics. However, this approach would require prior knowledge and characterization of tumor-specific antigens expressed on the tumor cells of each individual patient, and the use of an antibody specific for that particular type of tumor cell.

Summary of the Invention

The present invention employs various forms of beta-2 microglobulin to address the problems associated with failure of the cytotoxic T-cell activation pathway in both vaccination and tumor therapy. The invention also provides compositions and methods based on β_2m that are broadly applicable to achieve expression of any desired target protein on the surface of any mammalian cell.

In one embodiment, the invention provides fusion proteins comprising a first amino acid sequence and a second amino acid sequence, wherein the second amino acid sequence is a β_2-microglobulin. In particular applications, the first amino acid sequence may be a co-stimulatory protein, such as B7.1 or B7.2, or another protein having immunological activity, such as a cytokine, an integrin or a cellular adhesion molecule. Examples of such proteins include interleukins (e.g., IL-2, IL-12), granulocyte-macrophage colony-stimulating factor (GM-CSF), lymphocyte function-associated proteins (e.g., LFA-1, LFA-3) and intercellular adhesion molecules (e,g., ICAM-1, ICAM-2). In other embodiments, the first amino acid sequence of the fusion protein may be any protein that is desired to be expressed on the surface of a cell. It is shown that these fusion proteins are an effective way to target a desired protein, such as B7, to the outer membrane of a cell. ("B7" is used generically to refer to either B7.1 or B7.2).

With respect to tumor therapy, it is shown that expressing on the surface of a tumor cell a fusion protein comprising a β_2m joined to a co-stimulatory protein can significantly increase the immune response of an animal to the tumor cell. In one example, a fusion protein comprising hβ_2m joined to the co-stimulatory protein B7 (and termed B7-β_2m) is targeted to the surface of tumor cells, such that the tumor cells present the B7-β_2m fusion protein to T-cells. These cells are then attenuated and introduced into mice. T-cells removed from these mice were shown to be significantly more active against the same type of tumor cells than equivalent cells from mice treated with tumor cells presenting β_2m only.

The β_2m fusion proteins provided by the invention have wide application in that they are useful to target any desired protein to the outer membrane of a cell. These fusion proteins may be targeted to the surface of a cell in a number of ways. In one approach, cells that express MHC I are

simply incubated with a preparation of the fusion protein, resulting in the incorporation of the fusion protein onto the cell surface. Alternatively, the fusion protein may be introduced into the cell so that it is incorporated into the MHC I pathway. In another approach, a nucleic acid molecule encoding the fusion protein is introduced into a cell by transformation. Expression of this nucleic acid molecule results in the fusion protein being produced within the cell and exported to the cell membrane. Where the fusion protein is to be introduced into the cytosol for export to the outer membrane, or where it will be expressed by a nucleic acid molecule within the cell, it is desirable to include a signal peptide at the N-terminus of the fusion peptide so that the fusion protein is transported to the outer membrane of the cell. The β_2m signal peptide may be used for this purpose. In all of these approaches, the result is that the β_2m fusion protein is presented on the surface of the cell.

In one embodiment, the invention includes nucleic acid molecules encoding the disclosed fusion proteins, as well as nucleic acid vectors comprising such nucleic acid molecules. Transgenic cells comprising these nucleic acid molecules are also provided by the invention.

Methods of expressing a β_2m fusion protein on the surface of a cell are provided by the invention. Such methods include contacting a cell with a fusion protein comprising a first amino acid sequence and a second amino acid sequence wherein the second amino acid sequence is a β_2m. An alternative method provided by the invention comprises transforming the cell with a nucleic acid molecule encoding such a fusion protein.

The invention further provides methods of enhancing the immune response of an animal to an antigen presented on the surface of a cell. Such methods comprise providing a β_2m fusion protein on the surface of the cell and administering the cell to the animal. In such applications, the fusion protein preferably comprises β_2m fused to a co-stimulatory protein, such as B7, or another protein having immunological activity. Expressing the β_2m fusion protein on the surface of the cell may be accomplished by contacting the cell with the fusion protein, or transforming the cell with a nucleic acid molecule encoding the protein. These methods may be applied to the treatment of tumors; in such treatments, the antigen against which an enhanced immune response is desired is a tumor antigen, and the cell bearing the antigen is a tumor cell. The tumor cell may be removed from the body of a mammal having a tumor, or may be derived from an *in vitro* propagated tumor cell line. The β_2m fusion protein is introduced to the tumor cell (e.g., by incubation of the tumor cell with the protein, or by transformation of the tumor cell with a nucleic acid encoding the fusion protein), such that the tumor cell presents the fusion protein on its surface. The tumor cell carrying the fusion protein is then administered to a mammal. In certain embodiments, the tumor cell may be attenuated prior to being administered to the mammal; such attenuation may be accomplished using standard means such as radiation, heat or chemical treatment. Once in the body of the mammal, the combination of tumor antigens and the β_2m-fusion protein on the surface of the tumor cells are recognized by $CD8^+$ T-cells, resulting in T-cell activation, proliferation and thereby an enhanced

cytolytic T-cell response against both the introduced tumor cells and other tumor cells in the mammal that express the same tumor antigen.

The present invention also provides modified human β_2m (hβ_2m) proteins having an enhanced affinity for MHC I. Such proteins are shown to bind to the alpha chain of MHC I with higher affinity than wild-type hβ_2m and to enhance T-cell recognition of APCs bearing the modified hβ_2m. In particular embodiments, the modified hβ_2m proteins have a valine residue at position 55 in place of the serine residue that is found in the mature form of naturally occurring (i.e., wild-type) hβ_2m. Such modified hβ_2m proteins are referred to as hβ_2m S55V.

hβ_2m S55V is useful as a vaccine adjuvant in place of wild-type hβ_2m. Thus, one aspect of the invention is a vaccine preparation comprising at least one antigen and hβ_2m S55V. hβ_2m S55V may also be utilized in place of wild-type hβ_2m in the fusion proteins discussed above. Additionally, β_2m fusion proteins may also be employed in such vaccine preparations, either using a wild-type β_2m or, in the case of hβ_2m, hβ_2m S55V.

Sequence Listing

The nucleic and amino acid sequences listed in the Sequence Listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

Seq. I. D. No. 1 shows the amino acid sequence of wild-type (naturally occurring) hβ_2m.

Seq. I. D. No. 2 shows the amino acid sequence of the B7-β_2m fusion protein (comprising the B7.2 co-stimulatory molecule).

Seq. I. D. No. 3 shows the amino acid sequence of the B7-β_2m fusion protein having the β_2m signal sequence joined to the N-terminal of the B7 domain.

Seq. I. D. Nos. 4-7 show primers used to construct hβ_2m S55V.

Seq. I. D. Nos. 8 and 9 show primers used to amplify the B7.2 protein.

Seq. I. D. No. 10 shows the amino acid sequence of mature hβ_2m S55V.

Seq. I.D. Nos. 11 and 12 show the amino acid linker sequences that can be used between the two domains of a fusion protien.

Seq. I.D. Nos. 13 and 14 show amino acid sequences of signal peptides that can be used to direct the expression of a protein in a cell.

Seq. I.D. No. 15 shows the amino acid sequences for a c-myc tag.

Seq. I.D. Nos. 16-20 show the amino acid sequences for peptides used in the HLA stabilization assay.

Brief Description of the Drawings

FIG. 1 shows the amino acid sequence of naturally occurring (i.e., wild-type) $h\beta_2m$; the signal peptide is double underlined and the amino acid numbering starts at the isoleucine residue that is the first residue of the mature protein. The serine at position 55 that is changed to valine in S55V is shown in bold.

FIG. 2 is a schematic representation of the B7.2-β_2 microglobulin fusion peptide construct.

FIG. 3 shows the sequence of the B7.2-β_2m fusion peptide. Residue 1 is a methionine required for expression, residues 2-220 are the extracellular portion of murine B7-2, residues 221-225 (italics) are a sequence created by the insertion of a restriction site into the nucleic acid sequence, residues 226-240 (underlined) are the linker sequence, and residues 241-339 are the mature form of $h\beta_2m$.

FIG. 4 shows the sequence of a B7-β_2m fusion peptide having the $h\beta_2m$ signal sequence (residues 1-20, double underlined). Residues 21-239 are the extracellular portion of murine B7-2, residues 240-244 (italics) are a sequence created by the insertion of a restriction site into the nucleic acid sequence, residues 245-259 (underlined) are the linker sequence, and residues 260-358 are the mature form of $h\beta_2m$.

FIG. 5 is a graph showing the effect of plate-bound B7-β_2m on BALB/c splenic T-cell proliferation in the presence of a suboptimal concentration of soluble 2C11.

FIG. 6 is a graph showing the efficacy of P815 antigen-primed T-cells (primed with P815 cells presenting only P815 antigens, or P815 antigens and either B7-β_2m or β_2m) to lyse P815 tumor cells.

FIG. 7 is a graph illustrating the stabilization of cell-surface HLA-A1 (a), -A2 (b), and -A3 (c) by mutant $h\beta_2m$ and peptide. All values are expressed as mean fluorescence intensity.

FIG. 8 shows the inhibition of myc-β_2m binding by S55V and $h\beta_2m$ to cell-surface HLA-A1 (a), -A2 (b), and -A3 (c). All values are expressed as mean fluorescence intensity.

FIG. 9 illustrates that the S55V mutant enhances CTL recognition better than wild-type $h\beta_2m$ in both Hmy2.C1R-A2 (a) and Hmy2.C1R-A3 (b) target cells.

Detailed Description of the Invention

Abbreviations and Definitions

To facilitate review and understanding of the invention as described herein, the following explanation of abbreviations and definitions of terms are provided:

β_2m: beta-2 microglobulin. This term encompasses any mammalian beta-2 microglobulin protein, including human and murine beta-2 microglobulins. The term "$h\beta_2m$" refers specifically to human beta-2 microglobulin. cDNAs and genes encoding mammalian $\beta_2$ms are well known in the art, as are the corresponding β_2m protein sequences. Examples include those sequences described in: Parnes and Seidman (*Cell* 29:661-9, 1982), Gates et al. (*Proc. Natl. Acad. Sci. USA* 78:554-8, 1981)

(murine); Suggs et al. (*Proc. Natl. Acad. Sci. USA* 78:6613-7, 1981), Guessow et al. (*J. Immunol.* 139:3132-8, 1987), Cunningham et al. (*Biochem.* 12:4811-22, 1973) (human); and Ellis et al. (*Immunogenetics* 38:310, 1993) (bovine). These sequences are also available on GenBank at http://www.ncbi.nlm.nih.gov/Entrez/index.html.

The terms "wild-type β_2m" and "naturally occurring β_2m" refer to the β_2m protein that is isolated from the particular species of mammal in question. For example, wild-type hβ_2m refers to a beta-2 microglobulin protein having an amino acid sequence of β_2m isolated from a human source (e.g., serum). Thus, an example of a wild-type β_2m is the hβ_2m protein disclosed in Cunningham et al. (*Biochem.* 12:4811-4822, 1973), which is also available on GenBank under accession number A90371 and is shown in FIG. 1 and Seq. I.D. No. 1. The term "modified β_2m" refers to a beta-2 microglobulin protein having an amino acid sequence that has been modified from a wild-type β_2m amino acid sequence. By way of example, hβ_2m S55V is a mutant form of hβ_2m in which the serine residue present at position 55 in mature wild-type hβ_2m (see FIG. 1 and Seq. I.D. No. 1) is replaced with a valine residue. The term hβ_2m S55V encompasses forms of hβ_2m that differ from wild-type hβ_2m by the substitution of the position 55 serine for a valine, as well as forms of hβ_2m that have the S55V modification and additional amino acid sequence modifications.

Fusion protein: A protein comprising two amino acid sequences that are not found joined together in nature. The term "β_2m fusion protein" refers to a protein that comprises a first amino acid sequence and a second amino acid sequence, wherein the second amino acid sequence is a β_2-microglobulin. The β_2m amino acid sequence and the first amino acid sequence may alternatively be referred to as domains of the fusion protein. Thus, for example, the present invention provides fusion proteins comprising first and second domains, wherein the second domain is a β_2m protein. The link between the first and second domains of the fusion protein is typically, but not necessarily, a peptide linkage. In particular β_2m fusion proteins, the two domains may be joined by means of a linker peptide. In β_2m fusion proteins, the first domain is preferably, but not necessarily, linked to the N-terminus of the β_2m domain.

These fusion proteins may also be represented by the formula X-Y wherein X is a protein, such as a co-stimulatory protein, and Y is a β_2m protein. In a further embodiment of the fusion proteins disclosed, a signal peptide sequence may be linked to the N-terminus of the first protein. Such a three part protein can thus be represented as S-X-Y wherein S is the signal peptide, X is a protein, such as a co-stimulatory protein and Y is a β_2m protein. Where the fusion protein is being expressed in a eukaryotic cell, the signal peptide is preferably a eukaryotic signal peptide that functions to target expression of the fusion protein to the cell membrane. While a number of signal peptides may be used for this purpose, the preferred signal peptide is the β_2m signal peptide (shown in FIG. 1). Where the fusion protein is being expressed in a prokaryotic cell, the signal peptide is preferably a prokaryotic signal peptide that results in the secretion of the fusion peptide into the growth medium from where it can be readily harvested and purified. Suitable prokaryotic signal

peptides are well known in the art. Where the X protein and β_2m are joined by a peptide linker, the fusion protein may be represented as X-L-Y or, if a signal peptide is present, S-X-L-Y, where L is the linker peptide.

Certain β_2m fusion proteins of the present invention include, as their β_2m component, the $h\beta_2$m S55V protein. Particular residues in the β_2m component of such fusion proteins may be referred to by the number of residues that they are away from the first residue of the mature $h\beta_2$m (which is isoleucine). Thus, the β_2m component of a fusion protein that includes $h\beta_2$m S55V may be referred to as a human β_2-microglobulin having a valine at position 55.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Isolated: An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known in the art.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified β_2m protein preparation is one in which the β_2m protein is more pure than the protein in its natural environment within a cell. Preferably, a preparation of a protein is purified such that the protein represents at least 50% of the total protein content of the preparation.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated

segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

Tumor Cell: A neoplastic cell that may be either malignant or non-malignant. Tumor cells include cells from both solid and non-solid tumors (such as hematologic malignancies). Tumors may be primary tumors originating in a particular organ (such as breast, prostate, bladder or lung). Tumors of the same tissue type may be divided into tumors of different sub-types (a classic example being bronchogenic carcinomas (lung tumors) which can be an adenocarcinoma, small cell, squamous cell, or large cell tumor).

Mammal: This term includes both human and non-human mammals. Similarly, the term "patient" includes both human and veterinary subjects.

Production and use of β_2m fusion proteins

Standard molecular biology, biochemistry and immunology methods are used in the present invention unless otherwise described. Such standard methods are described in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989), Ausubel et al. (*Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences, 1987), Innis et al. (*PCR Protocols, A Guide to Methods and Applications*, Innis et al. (eds.), Academic Press, Inc., San Diego, California, 1990) and Harlow and Lane (*Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). Methods of producing nucleic acid sequences expressing fusion proteins, as well as methods of expressing and purifying fusion proteins are well known in the art, and are described, for example, in U.S. patent Nos. 5,580,756 and 5,698,679. The following paragraphs are provided by way of guidance.

Standard techniques may be employed to make genetic constructs expressing β_2m fusion proteins, including restriction endonuclease digestion, ligation and the polymerase chain reaction. Any mammalian gene or cDNA encoding β_2m may be used as the source of the β_2m coding sequence. Such sequences are known in the art and are available on public databases such as GenBank. By way of example, the sequence of the human β_2m cDNA is described in Guessow et al. (*J. Immunol.* 139:3132-8, 1987, GenBank accession number: M17986). Notably, this cDNA sequence includes regions coding for the signal peptide of hβ_2m (see FIG. 1).

Similarly, nucleic acid sequences coding for proteins that may be selected as the second domain of the fusion protein are well known in the art. While the selection of the second domain protein will typically be a co-stimulatory protein or a protein having some other immunological activity, fusion proteins may be constructed using β_2m as the second domain, and any protein that is desired to be delivered to the surface of a cell as the first domain. Examples of cDNAs encoding proteins having co-stimulatory activity include those encoding human B7.1 (Freeman et al., 1989, *J. Immunol.* 143:2714-22, GenBank accession number: M27533), B7.2 (Azuma et al., 1993, *Nature*

366:76-9, GenBank accession number L25259), LFA-3 (Wallner et al., 1987, *J. Exp. Med.* 166:923-32, GenBank accession number Y00636) and ICAM-1 (Simmons et al., 1988, *Nature* 331:624-7, GenBank accession number X06990). Examples of other immunologically active proteins include, ICAM-3 (Fawcett et al., 1992, *Nature* 360:481-4, GenBank accession number S50015), VCAM-1 (Damle et al., 1992, *J. Immunol.* 148:1985-92), CD59 (Menu et al., 1994, *J. Immunol.* 153:2444-56), CD40 (Hancock et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:13967-72) and GM-CSF (Takashi et al., JP 1991155798-A, GenBank accession number E02975). Proteins having other activities, such as tumor necrosis factor (TNF, Masaaki et al., JP 1985185799, GenBank accession number E00423) may also be employed as the first domain in the fusion protein. By way of example, proteins that induce apoptosis (such as TNF) or anergy may be employed to delete certain classes of antigen-specific activated T-cells. Thus, myelin basic protein (MBP)-specific autoreactive T-cells that are found at the site of inflammation in multiple sclerosis patients may be deleted by introducing into the patient target cells that express MBP and which also present a TNF-β_2m fusion protein.

cDNA clones encoding β_2m and the second protein may be obtained as described in the cited references, or by PCR amplification from mRNA (or cDNA libraries) of cells that express the particular protein. cDNA amplification is performed as described by Innis et al. (*PCR Protocols, A Guide to Methods and Applications*, Innis et al. (eds.), Academic Press, Inc., San Diego, California, 1990) using primers designed to amplify the desired portions of the cDNA. For example, cDNA primers may be designed to amplify only that portion of the β_2m cDNA that encodes the mature form of β_2m. PCR may also be used to adapt the amplified fragments for ligation.

In addition to a β_2m domain and a second protein domain (e.g., B7), β_2m fusion proteins may also include additional elements, such as a linker sequence between the β_2m domain and the second domain, and a signal peptide. The linker sequence is generally between 10 and 25 amino acids in length, and serves to provide rotational freedom in the fusion construct, thereby facilitating appropriate conformational folding of the two adjacent protein domains. Such linker sequences are well known in the art and include the glycine(4)-serine spacer (GGGGS x3, Seq. I.D. No. 11) described by Chaudhary et al. (*Nature* 339:394-397, 1989). A version of this linker in which the third repeat of the linker motif is modified to GGGAS (Seq. I.D. No. 12) is also shown in FIG. 3 and Seq. I.D. No. 2. Other linker sequences may also be used to construct the β_2m fusion proteins.

Signal peptides serve to direct expression of a particular protein to a specified location in the cell. Depending on whether the fusion protein is to be expressed in a prokaryotic or eukaryotic cell, a prokaryotic or eukaryotic signal peptide will be selected. Prokaryotic signal peptides that direct secretion of peptides into the medium may be particularly useful where large amounts of the fusion peptide are to be produced. Examples of such signal sequences include the prokaryotic signal sequence of the pectate lyase gene pelB (Power et al., 1992, *Gene* 113:95-9 (KYLLPTAAAGLLLLAAQPAMA, Seq. I.D. No. 13)), and the outer membrane protein ompT (Ouzzine et al., 1994, *FEBS Lett* 339:195-9 (MRAKLLGIVLTPIAISFAST, Seq. I.D. No. 14)).

Eukaryotic signal peptides that direct expression of a peptide to the cell surface are useful where the fusion protein is to be presented on the surface of the cell. The signal peptide of β_2m (shown in FIG. 3 and Seq. I.D. No. 2) is particularly suitable for this purpose.

In their most basic form, nucleic acids encoding β_2m fusion proteins will comprise x-y wherein x is a nucleic acid sequence encoding the first protein domain (e.g., B7) and y is a nucleic acid sequence encoding the β_2m protein domain. Where a linker sequence is to be included, the nucleic acid may be represented as x-l-y, wherein l is a nucleic acid sequence encoding the linker peptide. Where a signal sequence is to be included, the nucleic acid may be represented as s-x-l-y wherein s is a nucleic acid sequence encoding the signal peptide. Preferably, although not necessarily, the relative orientation of the nucleic acid sequences is such that in the encoded fusion protein, the N-terminal of the β_2m protein is linked to the C-terminal of the second protein domain. In all instances, the various nucleic acid sequences that comprise the β_2m fusion protein construct (e.g., s, l, x and y) are operably linked such that the elements are situated in a single reading frame.

Nucleic acid constructs expressing fusion proteins may also include regulatory elements such as promoters, enhancers and 3' regulatory regions, the selection of which will be determined based upon the type of cell in which the protein is to be expressed. The constructs are the introduced into a vector suitable for expressing the β_2m fusion protein in the selected cell type.

A selected β_2m fusion protein may be obtained by expression in a prokaryotic or eukaryotic expression system, many of which are well known in the art. Heterologous proteins can be produced in prokaryotic cells by placing a strong, regulated promoter and an efficient ribosome binding site upstream of the β_2m fusion protein construct. Suitable promoter sequences include the beta-lactamase, tryptophan (trp), and lambda derived P_L promoters. Prokaryotic expression vectors and expression systems suitable for producing high levels of protein bacterial cells are available commercially and include the pBAD, P_L and Superlinker expression systems produced by Invitrogen (Carlsbad, CA) and the pMAL expression system produced by New England Biolabs (Beverly, MA). In addition, methods and plasmid vectors for producing heterologous proteins in bacteria are described in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989). Often, proteins expressed at high levels are found in insoluble inclusion bodies; methods for extracting proteins from these aggregates are described by Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York, 1989, ch. 17). Vectors suitable for the production of intact native proteins include pKC30 (Shimatake and Rosenberg, 1981, *Nature* 292:128), pKK177-3 (Amann and Brosius, 1985, *Gene* 40:183) and pET-3 (Studiar and Moffatt, 1986, *J. Mol. Biol.* 189:113). Suitable prokaryotic cells for expression of large amounts of β_2m fusion proteins include *Escherichia coli* and *Bacillus subtilis*.

Eukaryotic cells such as Chinese Hamster ovary (CHO), monkey kidney (COS), HeLa, *Spodoptera frugiperda*, and *Saccharomyces cerevisiae* may also be used to express β_2m fusion proteins. Regulatory regions suitable for use in these cells include, for mammalian cells, viral

promoters such as those from CMV, adenovirus and SV40, and for yeast cells, the promoter for 3-phosphoglycerate kinase and alcohol dehydrogenase. Eukaryotic cell expression systems are also commercially available, and include *Pichia pastoris*, *Drosophila*, Baculovirus and Sindbis expression systems produced by Invitrogen (Carlsbad, CA).

The transfer of DNA into eukaryotic, in particular human or other mammalian cells, is now a conventional technique. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, 1973, *Virology* 52:466) or strontium phosphate (Brash et al., 1987, *Mol. Cell Biol.* 7:2013), electroporation (Neumann et al., 1982, *EMBO J* 1:841), lipofection (Felgner et al., 1987, *Proc. Natl. Acad. Sci USA* 84:7413), DEAE dextran (McCuthan et al., 1968, *J. Natl Cancer Inst.* 41:351), microinjection (Mueller et al., 1978, *Cell* 15:579), protoplast fusion (Schafner, 1980, *Proc. Natl. Acad. Sci. USA* 77:2163-7), or pellet guns (Klein et al., 1987, *Nature* 327:70). Alternatively, the nucleic acid molecules can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein et al., 1985, *Gen. Engr'g* 7:235), adenoviruses (Ahmad et al., 1986, *J. Virol.* 57:267), or Herpes virus (Spaete et al., 1982, *Cell* 30:295).

The β_2m fusion protein produced in mammalian cells may be extracted following release of the protein into the supernatant and may be purified using an immunoaffinity column prepared using anti-β_2m antibodies. Alternatively, the β_2m fusion protein may be expressed as a chimeric protein with, for example, β-globin. Antibody to β-globin is thereafter used to purify the chimeric protein. Corresponding protease cleavage sites engineered between the β-globin gene and the nucleic acid sequence encoding the β_2m fusion protein are then used to separate the two polypeptide fragments from one another after translation. One useful expression vector for generating β-globin chimeric proteins is pSG5 (Stratagene).

By way of example cDNA encoding a β_2m fusion protein with an N-terminal methionine to initate translation may be subcloned into pET21-d (Novagen) which directs recombinant protein to inclusion bodies. Alternatively, commercially available insect cell expression systems such as the Baculovirus Expression Vector System from Pharmingen (San Diego, CA) can be used for the combined expression and folding of β_2m and β_2m fusion proteins, where the expressed proteins will require only subsequent purification.

Proteins expressed as described above may be further purified by immunoaffinity column and used directly to treat mammalian cells. Alternatively, expression of a β_2m fusion protein in a mammalian cell may be obtained by introducing a vector carrying a nucleic acid sequence encoding the protein into the cell. Nucleic acid molecules encoding β_2m fusion proteins carrying a signal sequence, such as the β_2m signal sequence are particularly preferred for this purpose.

In one aspect of the invention, the β_2m fusion proteins are used as immunotherapeutics, and the mammalian cell is a tumor cell. In many cancer patients, tumor cells escape immune recognition by downregulating MHC and/or co-stimulatory molecule expression. Accordingly, one method of

treatment previously proposed is to remove tumor cells from a patient, introduce into the cells a co-stimulatory molecule such as B7 and then return the cells to the patient (see for example European Patent Application publication number EP 0 733 373 A2). Applying the discovery disclosed herein to those methods, introducing a β_2m fusion protein into tumor cells is expected to provide considerable benefit.

Obtaining β_2m fusion protein expression on the surface of tumor cells may be achieved either by directly incubating tumor cells with a purified preparation of the fusion protein, or by introducing into the cells a vector that expresses the fusion protein. All types of tumor are potentially amenable to treatment by this approach including, for example, carcinoma of the breast, lung, pancreas, ovary, kidney, colon and bladder, as well as melanomas and sarcomas.

Incorporation of the β_2m fusion protein onto the surface of tumor cells by incubation of the purified fusion protein with the cells, may be achieved by incubation of the tumor cells with the recombinant protein (1-5 μM) in serum-free medium for 2-16 hours. Additionally, tumor cells can be treated briefly with a low pH buffer (pH 2.5 to 3.5) to strip endogenous β_2m and peptide from cell-surface MHC I molecules followed by reconstitution with the β_2m fusion protein and relevant MHC I binding peptides for 1-16 hours.

Where a nucleic acid encoding the fusion protein is to be introduced into the tumor cell, the nucleic acid is preferably incorporated into a suitable expression vector. Suitable vectors include plasmid, cosmid and viral vectors, such as retroviruses, adenoviruses and herpesviruses. Disabled viruses, such as those described in U.S. Patent No. 5,665,362 may be employed for this purpose. Because of the high efficiency with which viral vectors infect mammalian cells, viral vectors are expected to offer advantages over other vector types. The vector is then introduced into the tumor cell by one of a range of techniques, such as electroporation, lipofection, co-cultivation with virus-producing cells, or other standard means. In a preferred embodiment, the tumor cells are cells removed from the patient to be treated, but the tumor cells may alternatively be cells from a tumor cell line, such as the human tumor cell lines available from the American Type Culture Collection (ATCC, Manassas, VA). If it is desired to screen the cells to select those into which the vector was introduced, this may be achieved by a number of means, including selecting for expression of the selectable marker if one is used, or screening for expression of the β_2m fusion protein on the surface of the cells. This latter procedure may be conveniently performed using a fluorescence activated cell sorter (FACS).

The tumor cells are subsequently administered to the patient in combination with a suitable carrier such as buffered water, saline, or glycine. In a preferred embodiment, where the tumor cells are cells originally removed from the patient, they are attenuated before being administered to the patient. An attenuated cell is one which is metabolically active but which is no longer able to proliferate. Methods for attenuating tumor cells are well known and include those described in EP 0 733 373 A2.

In an alternative embodiment, cell membranes from the tumor cells, which include the β_2m fusion protein, may be administered to the patient instead of intact tumor cells. A cell membrane preparation can readily be prepared by disrupting or lysing the cells using standard techniques, such as a French Press, freeze-thawing, or sonication. Following disruption of the cells, a membrane enriched fraction may be obtained by centrifugation.

The present invention also encompasses other immunotherapy methods for treating conditions such as cancer, including adoptive immunotherapy. As is known in the art, adoptive immunotherapy involves obtaining lymphoid cells exposed to a particular antigen, culturing those cells *ex vivo* under conditions whereby the activity of the cells is enhanced, and then administering the cells to an individual. The lymphoid cells are preferably T-cells removed from a cancer patient, for example T-cells from a draining lymph node. These T-cells are incubated with tumor cells removed from the patient which have been treated as described above so as to present a β_2m fusion protein on their cell surface. Accordingly, one aspect of the present invention is a form of adoptive immunotherapy in which the incubation of lymphoid cells *ex vivo* is performed in a medium containing tumor cells presenting a β_2m fusion protein prior to administration of the cells to a patient. The technical details of methods for obtaining lymphoid cells, *ex vivo* cultivation of such cells with immune stimulants, and administration to patients are known in the field and are described, for example in U.S. Patent Nos. 4,690,915 ("Adoptive immunotherapy as a treatment modality in humans"), 5,229,115 ("Adoptive immunotherapy with interleukin-7"), 5,631,006 ("Immunotherapy protocol of culturing leukocytes in the presence of interleukin-2 in a hollow fiber cartridge"), and 4,902,288 ("Implantable immunotherapy system using stimulated cells"), and references cited therein.

Production and use of hβ_2m S55V and β_2m fusion proteins in vaccine preparations

Methods for the production and use of β_2m as a vaccine adjuvant are known in the art and include those described in U.S. patent No. 5,733,550, which is incorporated herein by reference. Such methods may be applied for the use of hβ_2m S55V as well as β_2m fusion proteins in vaccine preparations as well as methods of vaccination.

β_2m fusion proteins may be produced as described above. Nucleic acid molecules encoding forms of hβ_2m carrying the S55V amino acid substitution may be produced using standard mutagenesis techniques, such as site directed mutagenesis, or by PCR as described in Example 4 below. The encoded hβ_2m S55V may be expressed in and purified from prokaryotic or eukaryotic expression systems, as described above for the β_2m fusion proteins.

β_2m fusion proteins and hβ_2m S55V may be used as adjuvants in vaccine preparations, in which case they may be combined with an antigen in a vaccine preparation, or they may be administered to a patient either shortly before or after administration of a conventional vaccine preparation. Preferably, the β_2m fusion protein or hβ_2m S55V is administered at the same location

and contemporaneously with the antigen preparation. Where a $h\beta_2m$ fusion protein is used as a vaccine adjuvant, the $h\beta_2m$ component of the protein may be a S55V form of $h\beta_2m$. The protein to which the β_2m is fused will preferably be a co-stimulatory protein such as B7.

Typically, the antigen administered to a patient in conjunction with a β_2m preparation of the present invention (i.e., a preparation of a β_2m fusion molecule or a S55V $h\beta_2m$) will be a peptide antigen that can bind to class I MHC molecules of the patient. Peptide antigens that may be employed include tumor antigens, as well as antigens from pathogenic organisms, including viruses and bacteria. Examples of suitable antigens include HIV gp120, sub-units of influenza nucleoprotein or hemagglutinin, and tumor antigens as discussed by Boon et al., (*Ann. Rev. Immunol.* 12:337-65, 1994); Finn (*Curr. Opin. Immunol.* 5:701-8, 1993) and Sligluff et al. (*Curr. Opin. Immunol.* 6:733-40, 1994). Such antigens may be isolated or extracted from an original source (e.g., tumor cells), or may be produced by recombinant means, or may be chemically synthesized. Vaccination may be accomplished by administering a single peptide antigen or a cocktail of antigens derived from one or more antigen sources.

The β_2m that forms the basis of the β_2m fusion protein or β_2m S55V used in the vaccine compositions and methods of the present invention may be any mammalian β_2m. It may be preferable to use a β_2m derived from the same species of mammal as the mammal to be vaccinated so as to reduce the risk of immune response to the administered β_2m preparation. However, since xenogeneic β_2m is typically not inflammatory *in vivo*, this may not be necessary.

Vaccine preparations according to the present invention may be administered by any known means, including intramuscular and intravenous injection. In its simplest form, the β_2m preparation administered to the mammal is administered in conventional dosage form, preferably combined with a pharmaceutical excipient, carrier or diluent. Suitable pharmaceutical carriers may be solids or liquids, and may include buffers, anti-oxidants such as ascorbic acid, other polypeptides or proteins such as serum albumin, carbohydrates, chelating agents and other stabilizers and excipients. Suitable solid carriers include lactose, magnesium stearate, terra alba, sucrose, talc, stearic acid, gelatin, agar, pectin, acacia and cocoa butter. The amount of a solid carrier will vary widely depending on which carrier is selected, but preferably will be from about 25 mg to about 1g per dose of active agent. Suitable liquid carriers include neutral buffered saline, optionally with suitable preservatives, stabilizers and excipients. The carrier or diluent may also include time delay material well known to the art such as, for example, glycerol distearate, either alone or with a wax. The foregoing examples of suitable pharmaceutical carriers are only exemplary and one of skill in the art will recognize that a very wide range of such carriers may be employed. Liposome-based delivery systems may also be employed to deliver β_2m preparations. Liposome-based systems, which may be employed to provide a measured release of the agent over time into the bloodstream, are well known in the art and are exemplified by the systems described in U.S. Patent Nos. 4,356,167 ("Liposome drug delivery systems"), 5,580,575 ("Therapeutic drug delivery systems"), 5,595,756

("Liposomal compositions for enhanced retention of bioactive agents") and 5,188,837 ("Lipospheres for controlled delivery of substances"), and documents cited therein.

The formulation of the β_2m preparation with a pharmaceutical carrier can take many physical forms, but is preferably a sterile liquid suspension or solution, suitable for direct injection. Preferably, the patient will be administered the β_2m preparation in a formulation as described above (i.e. in combination with a pharmaceutical carrier), wherein the formulation includes a clinically effective amount of the agent. In the context of vaccination, "a clinically effective amount" of the β_2m preparation is an amount sufficient to provide an enhancement of the immune response to the target antigen, i.e., to produce a cytotoxic T-cell response greater than would be presented absent administration of the β_2m preparation. Quantification of the immune response arising from a vaccination may be achieved in any standard way, e.g., lymphoproliferation in response to test antigen *in vitro* or lysis of target cells by specific cytotoxic T-lymphocytes.

It will be appreciated that a clinically effective dose of a β_2m preparation will vary depending upon the actual β_2m being used (e.g., whether it is a β_2m fusion protein or β_2m S55V alone), and the characteristics of the patient (age, weight, other medications being taken etc.). Thus, the assessment of a clinically effective dosage will ultimately be decided by a physician, veterinarian, or other health care worker familiar with the patient. Typically, administering a β_2m preparation to a mammal as a component of a vaccination regimen will involve administration of from about 10 ng to 1 g of β_2m preparation per dose, with single dose units of from about 10 mg to 100 mg being commonly used, and specific dosages of up to 1 mg or 10 mg also being within the commonly used range. The amount of antigen included in the vaccine preparation that employs a β_2m adjuvant will typically be the same as would be included in vaccine preparations without the β_2m adjuvant, although greater or lesser amounts of antigen may be employed as clinically appropriate.

Where the β_2m is administered to the mammal in a single preparation with the vaccine antigens, the preparation may be formulated simply by mixing a clinically effective amount of the β_2m with the antigen preparation. Vaccines comprising tumor antigens and a β_2m may be prepared from tumor cells which have been transformed to express the β_2m.

EXAMPLES

The following examples serve to illustrate the invention.

EXAMPLE 1

Production of β_2m-B7 fusion protein

The murine B7.2:human β_2m fusion protein (m$B7\beta_2$) was made in multiple steps as follows. The extracellular domain of murine B7.2 (Freeman et al., 1993, *J. Exp. Med.* 178:2185-92, GenBank accession number L25606) was amplified by PCR from the plasmid mB7-2 (from Richard Hodes, NIH) using the following two oligonucleotides:

mB7-2 5' PCR Oligo: AGGGTACCATGGTTTCCGTGGAGACGCAAGC (Seq. I.D. No. 8) and mB7-2 3' PCR Oligo: TCGAATTCATGATGCTAGCCCAATACGTTTGAGGAGATGG (Seq. I.D. No. 9) which have embedded restriction sites for cloning (**bold**): KpnI: GAATTC; NcoI: CCATGG; and BspH1: TGATCA.

The resulting PCR fragment was cut with NcoI and BspH1, and ligated as an amino terminal extension into an NcoI cut bluescript SK vector containing the signal peptide of $h\beta_2m$ (Guessow et al., 1987, *J. Immunol.* 139:3132-8, GenBank accession number: M17986), the c-myc tag EQKLISEEDLN (Zhou et al., 1996, *Mol Immunol.* 33:1127-34, Seq. I.D. No. 15), and full length $h\beta_2m$ (plasmid #267). The resulting construct (plasmid #392) was then cut with NheI to linearize it 5' of the myc sequence. Synthetic oligonucleotides encoding a $[gly4ser]_3$ spacer were engineered with NheI compatible ends and ligated into the linearized vector to create plasmid #396. Finally, the entire coding sequence of wild-type $h\beta_2m$ (without a myc tag) was PCR amplified from a $h\beta_2m$ cDNA, with the addition of NheI site 5' and a Not I site 3' of the coding sequence. This product was digested with NheI and NotI, and subcloned into plasmid #396 that had also been digested with NheI and NotI to generate plasmid #406. This plasmid contained the signal sequence of $h\beta_2m$, followed by the extracellular domain of mB7.2, a 15 amino acid spacer, then mature $h\beta_2m$. For expression in bacteria, the eukaryotic signal sequence was removed. Thus, plasmid #406 was digested with NcoI and NotI to liberate the fusion protein without the signal peptide present, and this was then subcloned into the bacterial expression vector pET21-d that had been linearized with NcoI and NotI.

Following transfection of the BL21 (DE3) strain of *E. coli*, protein synthesis is induced with IPTG, cells were havested, lysed, and inclusion bodies washed and solubilized. Following refolding of the recombinant material, it was further purified by gel filtration and/or affinity chromatography with anti-β_2m antibodies. Experiments in which acid-stripped cells expressing only HLA-A2 were incubated under various conditions with the B7-β_2m fusion protein produced as described above, and then subjected to FACS analysis using conformationally-sensitive antibodies of varying specificities confirmed the following: (1) that the B7 domain of the fusion protein is natively folded; (2) that the β_2m domain of the fusion protein is natively folded; and (3) that the β_2m domain of the fusion protein functions to stabilize MHC I expression (data not shown).

EXAMPLE 2

The B7-β_2m fusion protein co-stimulates T-Cells

The ability of B7-β_2m to co-stimulate splenic T-cells was determined *in vitro*. Antibodies specific for the B7 receptor CD28 were used as a control. β_2m, recombinant B7-β_2m, or anti-CD28 was added to the wells of a microtiter plate and incubated at 37°C for 2 hours to promote binding. Thereafter the plates were washed to remove excess reagent, and splenic T-cells from BALB/c mice were added to the wells in the presence of sub-optimal concentrations of the soluble anti-T-cell receptor 2C11. The plates were incubated for 48 hours and then ^3H-thymidine was added and

allowed to incorporate into the proliferating cells for 20 hours. At the end of the time period, the cells were removed and the amount of ^{3}H-thymidine taken up was measured.

The results of these experiments showed that while no T-cell proliferation was observed in wells containing β_2m alone, recombinant B7-β_2m provides co-stimulation to the T-cells at least as effectively as the anti-CD28 antibody. A typical result is shown in FIG. 5.

EXAMPLE 3

Treatment of tumor cells with the β_2m-B7 fusion protein boosts the generation of tumor-specific cytotoxic T-cells

The ability of the B7-β_2m fusion protein to stimulate T-cell recognition and response against tumor cell antigens was compared to the corresponding activity of hβ_2m alone. DBA/2 mice were each vaccinated with 3 x 10^6 syngeneic P815 tumor cells that had been previously incubated in serum-free Iscove's Modified Dulbecco's Medium (SF IMDM) with either 0.2 μM B7-β_2m, 0.2 μM hβ_2m alone, or no additional reagent. Samples of each type were analyzed by flow cytometry to show that they stained for B7 or hβ_2m. These tumor cells were then irradiated with 20,000 Rads and injected into the mice. One week later the mice were reimmunized with identically prepared cells. After an additional week, the mice were sacrificed and their spleen cells were restimulated in culture with irradiated P815 stimulator cells for one week. The resulting cultured T-cells were then assayed for ability to kill untreated P815 tumor cells.

The results, depicted in FIG. 6, show that the spleen cells from the mice immunized with the B7-β_2m fusion protein-treated tumor cells were three fold more effective at killing tumor cells than cells from mice immunized with either the hβ_2m treated or untreated tumor cells. These data show that the presence of the fusion protein on the tumor cell surface enhances the immune system response to the tumor antigens present on the tumor cell surface.

EXAMPLE 4

Production of high affinity variant of hβ_2m

A number of variant forms of hβ_2m were created and tested for activity using MHC stabilization, T-lymphocyte lysis and myc-hβ_2m binding and inhibition assays. The hydrophilic serine 55 (S55) residue of hβ_2m that is buried at the hβ_2m/heavy chain interface and is situated directly adjacent to an ordered water molecule was identified as the target residue for mutagenesis. This residue was mutagenized to hydrophobic residues of increasing mass (valine, isoleucine, phenylalanine) in order to promote hydrophobic interactions and exclude the ordered water.

The hβ_2m sequence variants were constructed by mutating hβ_2m cDNA in Bluescript SK (Stratagene, La Jolla, CA) using the ExSite mutagenesis system (Stratagene) according to the manufacturer's protocol. The mutated cDNAs were subcloned into the bacterial expression vector pET-21d(+) (Novagen, Madison, WI) using engineered NcoI and BamHI sites at the 5' and 3' end of

the mature protein sequence, respectively. Oligonucleotides used to create variants of the $h\beta_2m$ sequence were as follows:

Sense S55F: 5' TTC TTC AGC AAG GAC TGG TCT TTC 3' (Seq. I.D. No. 4)

Sense S55I: 5' ATT TTC AGC AAG GAC TGG TCT TTC 3' (Seq. I.D. No. 5)

Sense S55V: 5' GTG TTC AGC AAG GAC TGG TCT TTC 3' (Seq. I.D. No. 6)

Common antisense: 5' TAA GTC TGA ATG CTC CAC TTT TTC 3' (Seq. I.D. No. 7)

Expression and purification of the mutated $h\beta_2ms$ was performed as previously described by Shields et al. (*J. Immunol.* 160:2297-307, 1998). Briefly, $h\beta_2m$ constructs in pET-21d(+) were transformed into the BL21(DE3) strain of *E. coli*. At an $O.D._{600nm}$ of 0.6, cultures were induced with 1 mM IPTG for four hours, and inclusion bodies isolated by centrifugation after sonication of bacteria in 200 mM Tris, 2 mM EDTA, 10% Triton X-100, pH 7.6 and washing in 200 mM Tris, 2 mM EDTA, pH 7.6. Inclusion bodies were solubilized in 6 M Guanidine-HCl containing 0.3 M DTT, 100 mM Tris, pH 8.0, and a mixture of protease inhibitors (5 μg/ml Leupeptin, 0.5 mM AEBSF, 1% Aprotinin). Following overnight dialysis in 6 M Guanidine pH 2.0, recombinant protein was refolded over 72 h in 0.4 M Arginine, 5 mM oxidized glutathione, 100 mM Tris, 2 mM EDTA at 10°C. Following refolding, preparations were dialyzed exhaustively against 0.4 M Arginine, 100 mM Tris, 2 mM EDTA, pH 8.0 and then PBS at 4°C. Preparations were purified as a single peak by preparative FPLC on a Superdex 75pg gel filtration column (Pharmacia, Uppsala, Sweden), concentrated using Centriprep-3 concentrating units (Amicon, Beverly, MA), sterile filtered and concentrations calculated based on $O.D._{280nm}$ readings. Recombinant $h\beta_2m$ was judged to be ≥95% pure based on analysis by SDS-PAGE, and analytical FPLC.

EXAMPLE 5

$h\beta_2m$ S55V produces enhanced MHC I stabilization

An HLA stabilization assay was used to screen the $h\beta_2m$ variants. A number of HLA alleles, HLA-A1, HLA-A2, and HLA-A3, were analyzed in order to determine whether any of the point mutants exhibited allele-specific effects.

In this and the following experiments, cell lines, monoclonal antibodies (mAbs) and peptides used were as follows:

Cell Lines and antibodies: Hmy2.C1R cells (Storkus et al., 1987, *J. Immunol.* 138:1657) were stably transfected with HLA-A1, -A2, and -A3 as previously described (Winter et al., 1991, *J. Immunol.* 146:3508; DiBrino et al., 1993, *J. Immunol.* 151:5930). HLA-A2/HTLV-1 TAX 11-19 peptide-specific CTL clone N1218 and HLA-A3/Influenza NP 265-273 peptide clone 2G12 were isolated and restimulated as previously described by Biddison et al. (*J. Immunol.* 159:2018, 1997). All mAbs were used as culture supernatants grown in DMEM supplemented with 10% fetal calf serum, 20 mM HEPES, 2 mM L-glutamine, 1% non-essential amino acids, 1% Pen-strep, and 0.04 mg/ml of Gentamicin sulfate (complete DMEM). GAP.A3 (HLA-A3 specific), and BB7.5 (pan-

HLA-ABC specific) hybridomas were obtained from the American Type Culture Collection (Manassas, VA). The myc-specific 9E10 hybridoma has been previously described by Evan et al. (*Mol Cell Biol.* 5:3610-6, 1985). Unless otherwise noted all solutions used for cell growth were obtained from Biofluids (Rockville, MD).

Peptides: The peptides used were the HLA-A1 binding ornithine decarboxylase 309-317 (OD 309): SSEQTFMYY (Seq. I.D. No. 16); the HLA-A2 binding HTLV-1 TAX 11-19: LLFGYPVYV (SEQ. I.D. No. 17) and HIV gag 77-85: SLYNTVATL (Seq. I.D. No. 18); and the HLA-A3 binding pn2a.A3: KLYEKVYTYK (Seq. I.D. No. 19) and influenza NP 265-273: ILRGSVAHK (Seq. I.D. No. 20) (DiBrino et al., 1993, *Proc. Natl. Acad. Sci USA* 90:1508; DiBrino et al., 1994, *J. Immunol.* 152:620; Honma et al., 1997, *J. Neuroimmunol.* 73:7; Parker et al., 1992, *J. Immunol.* 149:3580; Parker et al., 1994, *J. Immunol.* 152:163; and Parker et al., 1995, *Immunol. Res.* 14:34). These peptides were purchased from Bachem (Torrance, CA) or provided by Dr. John E. Coligan (Natl. Inst. of Allergy and Infectious Diseases, NIH). All peptides were purified by reverse phase HPLC and were >95% pure as determined by analytical HPLC and mass spectrometry.

The MHC stabilization was done essentially as described previously (Bremers et al., 1995, *J. Immunol. Emphasis Tumor Immunol.* 18:77; van der Burg et al., 1995, *Hum. Immunol.* 44:189; Sugawara et al., 1987, *J. Immunol. Methods* 100:83) with minor modifications. Briefly, Hmy2.C1R cells (Storkus et al., 1987, *J. Immunol.* 138:1657) were stably transfected with HLA-A1, -A2, and -A3 as previously described (Winter et al., 1991; *J. Immunol.* 146:3508; DiBrino et al., 1993, *J. Immunol.* 151:5930). Hmy2.C1R-A1, -A2, and -A3 cells were washed twice with PBS, resuspended in 0.13 M citric acid, 66 mM Na_2HPO_4, pH 2.9 (pH 3.2 for A2 cells), for 90 seconds at 4°C, washed with two 50 ml changes of IMDM, and resuspended in SF IMDM (identical to SF DMEM using IMDM instead). 10^5 cells per well were added to a 96 well microtiter plate containing hybridoma supernatants, peptide, and $h\beta_2m$ dilutions in a total volume of 150 μl. HLA-A1 transfected Hmy2.C1R cells were combined with BB7.5 mAb and 10 μg/ml A1-binding OD 309 peptide. HLA-A2 transfected Hmy2.C1R cells were combined with BB7.5 mAb and 2.5 μg/ml A2-binding HIV gag peptide. HLA-A3 transfected Hmy2.C1R cells were combined with GAP.A3 mAb and 1.25 μg/ml A3-binding pn2a.A3 peptide. After a four hour incubation at 23°C, cells were washed twice with FACS buffer (PBS, 2 mg/ml BSA, 0.02% NaN_3) and stained with FITC-conjugated goat anti-mouse IgG (H+L) $F(ab')_2$ fragment (Cappel/Organon Teknika, Durham, NC) for one hour at 4°C. Cells were washed twice with FACS buffer and fixed in 1% formaldehyde in PBS followed by flow cytometric analysis on a FACScan II machine (Becton Dickinson, Mountain View, CA).

Figure 7 demonstrates the ability of the S55 variant $h\beta_2m$ to stabilize HLA-A1 (FIG. 7a), HLA-A2 (FIG. 7b), and HLA-A3 (FIG. 7c) in the presence of a specific binding peptide and an appropriate HLA-specific antibody. The S55V variant ("X" symbol in FIG. 7) stabilized HLA-A1 and HLA-A3 approximately 2-fold and 3-fold better respectively, than wild-type $h\beta_2m$ (diamonds) at a molar level, and effects on HLA-A2 stabilization by S55V were slightly better than those observed

with wild-type $h\beta_2m$. S55F (squares) was similar to wild-type $h\beta_2m$ for all alleles tested, while the effects of S55I (triangles) varied depending on the allele (better with HLA-A1 and worse with -A2 and -A3).

EXAMPLE 6

$h\beta_2m$ S55V binds to MHCI with a higher affinity that wild-type $h\beta_2m$

The antibodies used in the experiments in the previous Example were selected due to their dependence on both $h\beta_2m$ and peptide in order to detect "complete" molecules, i.e. heavy chain/$h\beta_2m$/peptide natively folded trimeric complexes. Since this binding assay requires the presence of an antibody in addition to $h\beta_2m$ and peptide (van der Burg, 1995, *Hum. Immunol.* 44:189), there was the possibility that the antibody itself exerted an effect that is specific for a particular $h\beta_2m$ mutant. Due to concerns regarding the potential contribution of the antibodies to the stabilization of cell-surface MHC I complexes, a binding inhibition assay was developed that directly measures the relative abilities of $h\beta_2m$s to bind to MHC I molecules. This assay format requires a labeled $h\beta_2m$ to measure the inhibition. Methods of labeling such as biotinylation and iodination are random reactions and create multiply labeled species needing further purification prior to use in a proper competition assay (Hochman et al., 1988, *J. Immunol.* 140:2322). However, endogenous labeling with an epitope tag creates an uniquely labeled species of $h\beta_2m$. Additionally, tyrosine and lysine residues (common targets of biotinylation and iodination) known to be at the MHC heavy chain/$h\beta_2m$ interface would not be affected with an endogenous label. Therefore an epitope tag (myc) was engineered onto the amino terminus of $h\beta_2m$ and the ability of the various $h\beta_2m$ mutants to compete with the myc-$h\beta_2m$ for cell-surface binding using the anti-myc mAb 9E10 was studied.

To establish the functional activity of the myc-$h\beta_2m$ itself, direct binding studies were performed. Briefly, Hmy2.C1R transfectant cells at 2.5×10^5 per tube in a 500 μl volume were incubated at 37°C for 16 hours in SF IMDM with 2.5 μM myc-β_2m, 20 μg/ml peptide and the indicated concentrations of inhibitor β_2m. The OD 309 peptide was used for HLA-A1, the HIV gag peptide for HLA-A2, and the pn2a.A3 peptide for HLA-A3. Cells were washed three times in plain IMDM followed by incubation with 9E10 (anti-myc) hybridoma supernatant at 4°C for one hour. After washing with IMDM, cells were stained for one hour with FITC anti-mouse IgG at 4°. Cells were washed a final time in FACS buffer and analyzed by flow cytometry, gating on live (propidium iodide-excluding) cells. In the presence of an appropriate peptide there was concentration-dependent myc-β_2m binding for all alleles studied. However, when cells were incubated with myc-β_2m in the absence of peptide, no appreciable myc-$h\beta_2m$ binding was observed.

The relative abilities of wild-type $h\beta_2m$ and S55V to inhibit the binding of myc-$h\beta_2m$ to HLA molecules were next compared using an inhibition assay. The inhibition assay was identical to the binding assay with the following modifications: 2.5 μM myc-$h\beta_2m$ was used in all cases and

different concentrations of non-myc labeled recombinant $h\beta_2m$ were included to inhibit myc-$h\beta_2m$ binding to cell surface MHC molecules. Percent inhibition was calculated by the following equation: (1-((experimental-background)/(no inhibitor-background))) x 100. 10-20,000 gated events per sample were counted, and all experiments were repeated at least twice. Compared with wild-type $h\beta_2m$, the S55V mutant inhibited myc-$h\beta_2m$ binding about 2.5-fold better at a molar level for HLA-A1, -A2, and -A3 (FIG. 8). These results demonstrate the higher relative affinity of the S55V mutant compared to wild-type $h\beta_2m$ for HLA-A1 (FIG. 8a), -A2 (FIG. 8b) and -A3 (FIG. 8c).

EXAMPLE 7

$h\beta_2m$ S55V enhances CTL recognition of target cells

The effectiveness of $h\beta_2m$ in facilitating exogenous peptide loading of MHC I molecules was measured using a CTL lysis assay (Depierreux et al., 1997, *J. Immunol. Methods* 203:77) as follows: Hmy2.C1R cells transfected with HLA-A2 or HLA-A3 were resuspended to 4 x 10^6 cells/ml in complete DMEM supplemented with 20 μM BATD (which forms a fluorescent chelate with Europium; Wallac, Gaithersburg, MD) and incubated at 37°C for 30-60 minutes. Cells were resuspended in 10 ml of serum free (SF) CTL medium (IMDM supplemented with 5 mg/ml bovine serum albumin (Sigma, St. Louis, MO), 2 mM L-glutamine, 1.25 mM sulfinpyrazone (Sigma), and 1% Pen-strep), centrifuged and washed once more with SF CTL medium. Cells were then pulsed with peptide and/or β_2m in SF CTL medium for 60-90 minutes at 37°C. Cells were washed twice in SF CTL medium, resuspended in CTL medium (5% fetal calf serum in lieu of BSA) and combined at the designated effector:target ratio with CTL clones in round bottom microtiter plates (CTL clone N1218 at an E:T ratio of 4:1 (FIG. 9a) or the NP-specific HLA-A3 restricted CTL clone 2711 at an E:T ratio of 2:1 (FIG. 9b)). Plates were gently centrifuged at 100 x g for 2 minutes and then incubated at 37°C for 2 hours. Finally, plates were centrifuged at 300 x g and 20 μl per well was transferred to 200 μl of 0.3 M acetic acid, 60 mM sodium acetate, 7.5 μg/ml Europium (Aldrich, Milwaukee, WI), and the plate read on a Wallac 1234 DELFIA Fluorometer. Percent specific lysis was calculated with the following equation:

100 x ((experimental-blank)-(spontaneous-blank))/((maximum-blank)-(spontaneous-blank)).

In this assay, target cell lysis not only correlates with the loading of a specific peptide antigen, but it also demonstrates that the peptide is bound in an immunologically relevant manner. Hmy2.C1R-A2 target cells (a human lymphoblastoid cell essentially null for HLA molecules except for the transfected HLA-A2.1) (Winter et al., 1991; *J. Immunol.* 146:3508; DiBrino et al., 1993, *J. Immunol.* 151:5930) were pulsed with a suboptimal concentration of HTLV-1 TAX peptide (9.3 x 10^{-12} M/l) or control A2-binding HIV gag peptide at 1 x 10^{-9} M/l for 90 minutes in serum-free CTL medium in the absence or presence of increasing concentrations of purified, recombinant $h\beta_2m$ and then used as targets in a conventional lysis assay. The presence of wild-type $h\beta_2m$ dramatically increased the specific lysis by the TAX-specific CTL clone in a dose-dependent fashion. Using this

suboptimal concentration of peptide there was 20% lysis in the absence of $h\beta_2m$. Addition of 8 μM $h\beta_2m$ increases the lysis to the maximal observed at this E:T ratio. In the absence of $h\beta_2m$ 100 fold higher concentration of peptide would be required to achieve comparable levels of lysis (data not shown).

Having established the ability of wild-type $h\beta_2m$ to enhance the loading of antigenic peptide onto cells, the activity of the S55V variant in this assay was examined. Two CTL clones, specific for an HTLV-1 TAX peptide in the context of HLA-A2 and an influenza nucleoprotein peptide in the context of HLA-A3, were used in the assay described above using Hmy2.C1R transfectants pulsed with a suboptimal concentration of antigenic peptide (NP 265-273, 1×10^{-10} M/l) or control A3-binding pn2a.A3 peptide at 1×10^{-6} M/l. The S55V mutant was 4-fold more effective at a molar level than wild-type $h\beta_2m$ at enhancing target cell lysis for HLA-A2 (FIG. 9a) and 6 to 7-fold better for HLA-A3 (FIG. 9b). Controls with irrelevant -A2 and -A3 binding peptides with the highest concentrations of $h\beta_2m$ used resulted in only background levels of killing. Additionally, multiple TAX-specific A2-restricted clones displayed similar levels of S55V enhanced killing relative to wild-type β_2m (data not shown).

In view of the many possible embodiments to which the principles of the invention may be applied, it will recognized that the foregoing examples are offered for purposes of illustration and do not limit the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.